Growth Characteristics and Differentiation of Basal Cell Carcinoma In Vitro—Immunohistochemical, Gel Electrophoretic, and Ultrastructural Analysis

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Cell cultures were established from 48 solid basal cell carcinomas (BCC) and from the normal epidermis of the same patients. The growth characteristics and differentiation of BCC cells in vitro were compared with normal keratinocytes (nKC) by using immunohistochemistry, two-dimensional gel electrophoresis including immunoblots, transmission electron microscopy, and soft agar suspension culture. After isolation of the tumor tissue under a stereodissection microscope, explants were cultured on feeder layers of mitomycin-treated 3T3 cells. After 3–5 d, 73% of all explants of BCC could be successfully cultured showing spindle-shaped outgrowing cells. Compared to nKC, cultured BCC cells had a lower growth rate and showed a wider intercellular polymorphism regarding size and shape. Their labeling pattern with a wide panel of monoclonal antibodies showed significant differences from that of nKC. In particular, only weak reactions for various cytokeratins, filaggrin and vimentin depending on the BCC cell type (small, middle, large) were found. Two-dimensional gel electrophoresis revealed expression of keratins 5, 6, 14, 16, and 17 in BCC cells and of K 5, 6, 13, 14, 16, 17, and 19 in nKC. These findings were confirmed by immunoblot. On the ultrastructural level, only a few desmosomes and a lower degree of keratinization markers were detected in BCC cells; finally, when cultured in soft agar BCC cells formed colonies whereas nKC did not.

Our findings indicate that cultured BCC cells may preserve in vitro some in vivo characteristics and maintain a growth and differentiation pattern that differs from cultured nKC. The culture model presented here provides further insights into the cytogenetic and histogenetic characteristics of BCC. *J Invest Dermatol 99:474–481, 1992*

Basal cell carcinoma (BCC) is one of the most common skin neoplasms in humans, showing a characteristic growth and differentiation pattern [1,2]. Clinically, BCC grow slowly and metastasis rarely occurs. On the other hand, BCC cells are able to invade and to destroy the surrounding tissue. Histologically, BCC consist of basaloid cells resembling basal cells of the epidermis. Therefore, BCC was originally regarded to arise from the basal cell layer of the epidermis [3], but other early studies suggested that BCC may derive from primary epithelial germ cells, which may show differentiation towards skin appendages [4]. In a previous study using immunohistochemical and biochemical techniques, we reported on marked differences between BCC and basal cells of normal epidermis with regard to their keratin pattern in vivo [5]; some cytokeratins representing markers for keratinizing epithelia were not detectable in BCC.

In vitro culture techniques are now accepted to provide valuable models for experimental studies on the pathogenesis and histogenetic properties of skin tumors such as squamous cell carcinomas [6] and malignant melanomas [7]; these tumors have been frequently maintained in culture. Cell cultures of BCC were difficult to obtain so far, and there are only a few reports on the successful cultivation of BCC in vitro [8–11]. This may reflect some of the peculiar characteristics of BCC.

In the present study, we report on a culture technique for establishing explant cultures of BCC on 3T3 cell feeder layers that enabled us to obtain long-term primary cultures of nodular BCC. In this culture model, BCC cells showed distinct morphologic, ultrastructural, and cytochemical features that clearly differed from those of cultured epidermal keratinocytes.

MATERIALS AND METHODS

Tissues Biopsies from 48 nodular basal cell carcinomas were obtained. Histologically solid BCC types were found. The tumors had been excised from 22 female and 26 male patients, aged from 54 to 84 years, attending the Department of Dermatology at the Steglitz

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Abbreviations:
BCC: basal cell carcinoma
DMEM: Dulbecco's modified Eagle's medium
EGF: epidermal growth factor
EDTA: ethylenedinitrilotetraacetic acid
FCS: fetal calf serum
K: keratin
MoAb: monoclonal antibody
NEpHGE: nonequilibrium pH gradient electrophoresis
nKC: normal human keratinocytes
PBS: phosphate-buffered saline
SD: standard deviation of the mean
SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TBS: Tris-buffered saline

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Table I. Monoclonal Antibodies Used in This Study, Their Specificity, Commercial Source, and Working Dilution (APAAP)

MoAb	Specificity	Source	Dilution
PKK2	K 7,16,17,19	Labsystems	1:200
KL1	K 1,2,5,6,7,8,10,11,17	Immunotech	1:500
CK8.12	K 13,16	Biomakor	1:100
CK8.13	K 1,5,6,7,8,10,11,18	Biomakor	1:100
CK14	K 14	Biotex Medics	1:100
CK8.60	K 10,11	Biomakor	1:200
CK1	K 1	Enzo	1:100
CK10	K 10	Monosam	1:100
RPN1160	K 18	Amersham	1:200
RPN1162	K 7	Amersham	1:200
RPN1165	K 19	Amersham	1:200
RPN1166	K 8	Amersham	1:200
Filaggrin	Filaggrin	Paesel & Lorei	1:100
Vimentin	Vimentin	Labsystem	1:75
Laminin	Laminin	Immunotech	1:30
GB3	Basement membrane	Sera-Lab	1:125
Ki67	Proliferating cells	Dakopatts	1:40

Medical Center of the Free University of Berlin. Forty-six BCC were located on the face and scalp, one on the forearm, and one on the leg, respectively. Normal skin obtained from the same body area of each patient was used as control.

After excision, BCC and normal skin were trimmed of excessive connective tissue and washed in Ca^{++}- and Mg^{++}-free phosphate-buffered saline (PBS, pH 7.2, Seromed, Berlin, Germany).

Cell Culture All samples were immediately immersed for 1 h in Dulbecco's modified Eagle's medium (DMEM) containing 500 IU/ml penicillin and 500 μg/ml streptomycin (Seromed).

BCC Cells: After disinfection, the BCC were cut into small pieces, and tumor nodules were isolated with fine needles using a stereomicroscope. The isolated BCC-specimens, about 1.0 mm in diameter, were placed onto the bottom of 35-mm plastic Petri dishes. Mitomycin C–treated fibroblasts (Flow Laboratories, Meckenheim, Germany) were used for feeder layers, seeded in a density of 10^4 cells/cm^2 [12]. 0.5 ml complete medium comprised of DMEM containing 10% fetal calf serum (Seromed), 2% human Ab serum, 10^{-9} M choleratoxin (Calbiochem, Frankfurt, Germany), 0.4 mM glutamine (Seromed), 10 ng/ml epidermal growth factor (EGF, Serva, Heidelberg, Germany), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.5 μg/ml amphotericin B (Seromed) was added to the dishes. Human serum was used after decomplementation (56°C, 1 h) and filtration.

Normal Human Keratinocytes (Control): Small pieces of normal skin were incubated in 2.4 U/ml dispase (Boehringer, Mannheim, Germany) in PBS for 20 h at 4°C. The epidermal portions of the fragments were then separated from the dermis, dissected into pieces of 1.0 mm diameter, and explanted on a 3T3 cell feeder layer in 35-mm Petri dishes as described above. Cell culture was carried out at 37°C in a 5% CO_2, water-saturated atmosphere for 40 d. The medium was changed every third day. For statistical analysis of the data, the Student t test was used.

Five confluent dishes of BCC cells and nKC derived from five different donors were used for immunohistochemical study, and the other five confluent dishes were used for keratin analysis.

Immunohistochemistry Cells were detached by 0.25% trypsin (Seromed) and 0.02% EDTA in PBS and were diluted at 5×10^4 cells/ml in medium. Cells were transferred to glass slides by cytocentrifugation (Cytospin) and were dried at room temperature. They were fixed in acetone for 10 min and were processed for immunohistochemical staining using the alkaline phosphatase–anti-alkaline phosphatase (APAAP) technique [13]. The MoAb used in this study are listed in Table I. Cytocentrifuge preparations were incubated with primary MoAb, diluted in Tris-buffered saline (TBS, pH 7.4, 0.05M), with rabbit anti-mouse IgG (Dako, Glostrup, Denmark), and with the APAAP complex for 30 min, respectively. The procedure was repeated with an incubation period of 10 min. The reaction was visualized with new fuchsin, naphthol AS-BI, and Levamisole. The slides were counterstained with Mayer's hematoxylin and were evaluated under a light microscope. The slides stained with MoAb were scored for the percentage of positively stained cells in the following manner: −, 0–5%; +, 6–25%; ++, 26–50%; +++, 51–75%; ++++, 76–100%.

Electron Microscopy For transmission electron microscopy, 16-day–old BCC or nKC cultures grown on 35-mm plastic Petri dishes were washed twice with PBS and were fixed for 30 min at 4°C in a solution of 2.5% glutaraldehyde in 0.1 M cacodylate-HCl buffer, pH 7.4, as previously described [14]. The post-fixation was performed for 30 min at 4°C in a solution of 1% OsO_4 and 1.5% potassium ferrocyanide [15] in 0.1 M cacodylate-HCl buffer. After a 30-min block staining with 0.5% uranylacetate in barbital-acetate buffer (pH 6.1), the specimens were dehydrated and embedded in EPON as described [16]. After polymerization, the plastic Petri dishes were mechanically removed, the specimens were cut into pieces, and were re-embedded and further processed according to a previously described method [17]. Thin sections were contrasted with lead citrate and uranyl acetate and examined in a Zeiss 10 CR electron microscope.

Gel Electrophoresis

Cytoskeletal Preparations: Keratin-enriched protein fractions were obtained as described earlier [18–20]. Briefly, the 3T3 feeder layer was selectively removed from confluent cultures with 0.02% EDTA in PBS (pH 7.2, Seromed), and the cultured cells were trypsinized (0.25% trypsin in PBS) and harvested into a centrifuge tube. The cells were then homogenized by ultrasonic disruption in 20 mM Tris/HCl, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride and 5 μg/ml each of pepstatin and antipain (Sigma, St. Louis), and extracted twice with 600 mM KCl, 5 mM ethylenedinitrilotetraacetic acid (EDTA), 50 mM Tris/HCl, pH 7.4, 1% Triton X-100, and proteinase inhibitors. Ultrasonic disintegration and centrifugation at 20,000 × g for 12 min was performed in each extraction step. The resulting pellets, highly enriched in cytoskeletal proteins, were solubilized in sample buffer for gel electrophoresis.

Two-Dimensional Gel Electrophoresis and Immunoblot Analysis Two-dimensional analysis by non equilibrium pH gradient electrophoresis (NEpHGE) [21] and SDS-PAGE [22] as well as immunoblotting of separated polypeptides were performed as described elsewhere [23]. Gels were stained in 0.2% Serva Blue R.

For immunoblot analysis, proteins from unstained gels were electrophoretically transferred onto nitrocellulose paper [23]. After blocking of unspecific protein binding sites with 0.14 M TBS (10 mM Tris/HCl, pH 7.4, and 140 mM NaCl) supplemented with 0.05% Tween 20 and 15% horse serum (Seromed), the membrane was incubated overnight with antikeratin antibodies. Secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (Bio-Rad, Munich, Germany) being developed with 4-chloro-1-naphthol and hydrogen peroxide as described [24].

The antikeratin-antibodies used were the commercially available monoclonal antibody (MoAb) CK4.62 recognizing K19 and K13 (ICN, Eschwege, Germany), and the rabbit polyclonal antisera 8-2/4 and 10-2/2 discriminating the acidic (type I) and the neutral-to-basic (type II) keratin subfamilies, respectively [19,24].

Agar Suspension Culture After excision, the samples were immersed in an aqueous solution of poly (1-vinyl-2-pyrrolidon)-iodine complex (B. Braun, Melsungen, Germany) during 1 h for disinfection. BCC cells were mechanically isolated by filtration through a nylon mesh. Normal skin was incubated with 2.4 U/ml dispase for 20 h at 4°C, and the peeled epidermis was pipetted to obtain cell suspensions.

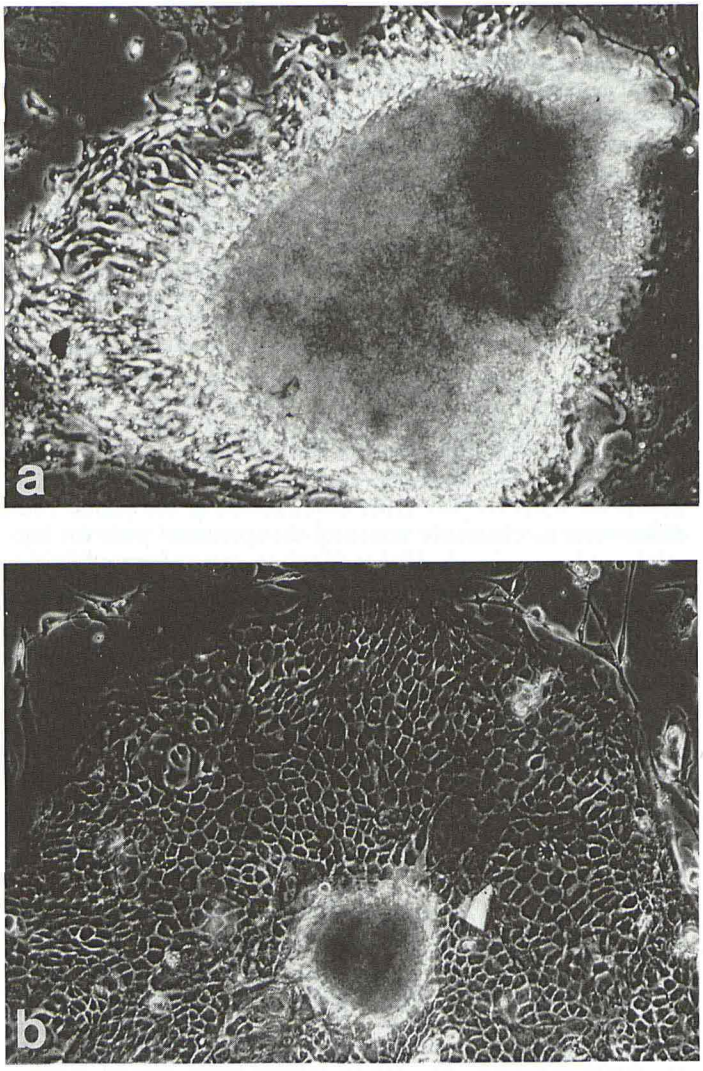

Figure 1. Explant cultures derived from basal cell carcinoma and normal epidermis on a 3T3 feeder layer (7 d old). BCC cultures show 2–3 rows of irregularly shaped, vacuolated cells *(a)*. Cultures of normal epidermal keratinocytes show 6–7 rows of uniform cells *(b)*.

The cell suspensions were diluted to 10^2 cells/ml in the medium used for explant cultures. 0.8 ml of each cell suspension was mixed with 0.2 ml of 3% agarose (Difco, Michigan) and immediately transferred into 35-mm Petri dishes.

One milliliter of the medium was added on the soft agar, and culturing was carried out under the same conditions as described for the explant cultures. In total, agar suspension cultures were initiated from 30 BCC and from five normal skin biopsies.

RESULTS

Morphology BCC cells were obtained from well-circumscribed nodular BCC by simple dissection using fine needles, whereby some BCC lacking a nodular solid growth pattern needed preparation under the stereo microscope. Thirty-five of 48 BCC explants (73%) and 44 of 48 nKC explants (92%) showed outgrowth of cells onto the substrate.

After 3 d of primary culture, only a few polygonal epithelial cells were seen surrounding the BCC explant, whereas there were already 3–4 rows of nKC at the same time. After 7 d, the cultures of BCC were composed of densely packed spindle-shaped cells (Fig 1*a*). They were vacuolated and irregular in size and shape, in contrast to nKC, which showed a more regular morphology

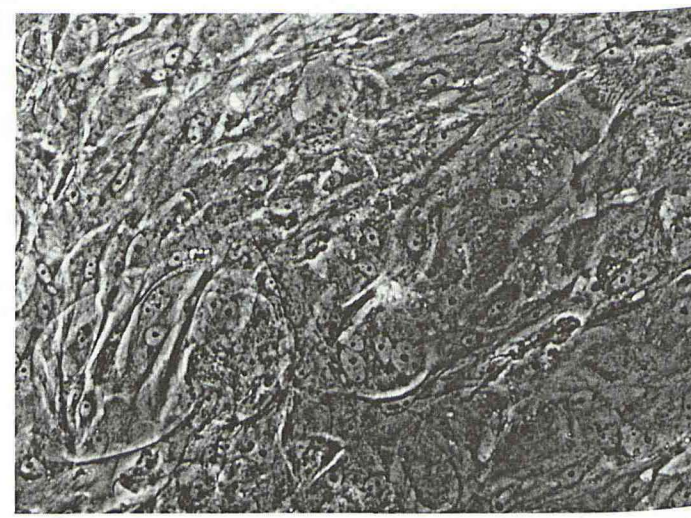

Figure 2. Basal cell carcinoma explant culture at confluence. Cells show irregularities in size and shape (magnification ×200).

(Fig 1*b*). After confluence, BCC cells produced marked variations in size and shape showing large nuclei (Fig 2) and limited stratification.

nKC became confluent after 14–18 d of primary culture; 16.2 ± 1.31 (mean ± SD, n = 9). In BCC cultures, confluency was reached in 10 of 35 dishes after 27–35 d, 29.2 ± 2.51 (mean ± SD, n = 10) ($p < 0.01$) (Table II).

Immunohistochemical Findings Immunohistochemical evaluation of cytospin-preparations of cultured BCC cells revealed marked variations in size, morphology, and staining patterns to the MoAb. According to the observed differences, at least three types of cells were thus discriminated by us and their immunohistochemical features, compared to nKC, are given separately in Table III. Overall, BCC cells were strongly stained with MoAb PKK2, KL1, CK8.12, CK8.13, and CK14; however, there were remarkable differences in the staining pattern of the different BCC cell types: small cells were stained with these MoAb in a way similar to nKC, in contrast to middle and large BCC cells. With the MoAb PKK2, small cells were strongly stained, whereas middle-sized cells showed an inconsistent, weak expression, and large cells were poorly stained (Fig 3*a*).

nKC were regular in size, and all strains showed a homogeneous staining pattern with the examined antibodies (Fig 3*b*). On the

Table II. Explant Cultures of BCC Cells and nKC; Comparison of the Time (d) Until Confluency Was Reached

Case	Age	Sex	Location	Days Until Confluency	
				BCC	nKC
1	54	M	Face	27	15
2	60	F	Face	29	14
3	61	M	Face	30	17
4	66	M	Face	28	16
5	67	M	Face	28	17
6	69	M	Face	28	15
7	73	F	Lower leg	33	16
8	78	F	Face	30	18
9	78	F	Forearm	31	18
10	84	F	Face	35	—[a]
Mean ± SD				29.9 ± 2.51 $p < 0.01$	16.2 ± 1.31

[a] No outgrowth observed.

Table III. Immunohistochemical Characterization of Cultured BCC Cells as Compared to nKC[a]

MoAb	BCC Cells			nKC
	Small	Middle	Large	
PKK2	++++	+++	+	++++
KL1	++++	++++	+++	++++
CK8.12	+++	++	+++	++++
CK8.13	++++	++	++	++++
CK14	+++	++	+	+++
CK8.60	−	(+)	−	++
CK1	−	−	−	(+)
CK10	−	−	−	−
RPN1160	−	−	−	−
RPN1162	−	−	−	−
RPN1165	−	(+)	−	++
RPN1166	−	−	−	−
Filaggrin	−	−	(+)	+++
Vimentin	+	(+)	−	+++
Laminin	++	+	−	+++
GB3	++	+	−	+++
Ki67				
Nuclear	++	+	+	(+)
Cytoplasmic	+	++	+++	+

[a] −, 0% cells stained; (+), 1–5%; +, 6–25%; ++, 26–50%; +++, 51–75%; ++++, 76–100%.

other hand, MoAb CK8.60, CK1, CK10, RPN1160, RPN1162, RPN1165, RPN1166, and Vimentin reacted only weakly with BCC cells or not at all. Differences were also found regarding MoAb CK8.60, RPN1165, and Vimentin (Table III). Filaggrin was generally not detectable in the small and middle types of BCC cells; however, a few large cells reacted with this antibody in a diffuse cytoplasmic pattern (Fig 4*a*). In nKC, filaggrin was expressed in the majority of the cells (51–75%), mostly in a granular pattern (Fig 4*b*). Laminin and GB3 stained small BCC cells, but not large cells, whereas strong reactions were observed with nKC. With MoAb Ki67, a nuclear pattern was found in a high number of small BCC cells, whereas a cytoplasmic pattern was observed in large cells, more pronounced than in nKC (Fig 5*a,b*).

Electron Microscopy After 16 d in primary culture, BCC cultures were composed of five to six keratinocyte layers (Fig 6*A*). The basal cells were thicker than the upper layers and contained large nuclei. Mitochondria, rough endoplasmic reticulum, Goldi complexes, and free ribosomes were regularly found in all cultured cells. However, hemidesmosomes were not detected, and desmosomes were rarely found, whereas cell to cell contacts consisted mainly of tight junctions (Fig 6*B*). Scattered intracytoplasmic tonofilaments of 6 to 8 nm diameter were only sparsely distributed, mainly arranged parallel to the cell membranes. BCC cultures did not show signs of further differentiation in vitro, as keratohyalin granules, multilamellar bodies, cornified envelopes, or superficial cornified cells were not detected (Fig 6*A*). In some cells, accumulations of irregularly arranged tonofilament bundles were found (Fig 6*B*).

Control cultures of nKC also showed stratification with 6 to 8 cell layers. The basal and intermediate cells contained regular cell organelles that were mainly arranged parallel to the cell membrane. In contrast to BCC cells, a considerable number of intercellular desmosomes were detected in the intermediate cell layers (Fig 7), whereas keratohyalin granules were not found. The uppermost cells showed signs of a corneocyte-like morphology with formation of cornified envelopes and absence of cell organelles.

Keratin Expression Pattern in BCC and nKC The two-dimensional gel electrophoretic separation of cytoskeletal proteins prepared from BCC cultures and nKC are shown in Fig 8*a,b*. The BCC cultures displayed few polypeptides on Coomassie Blue–stained gels that could be identified as keratins in the corresponding

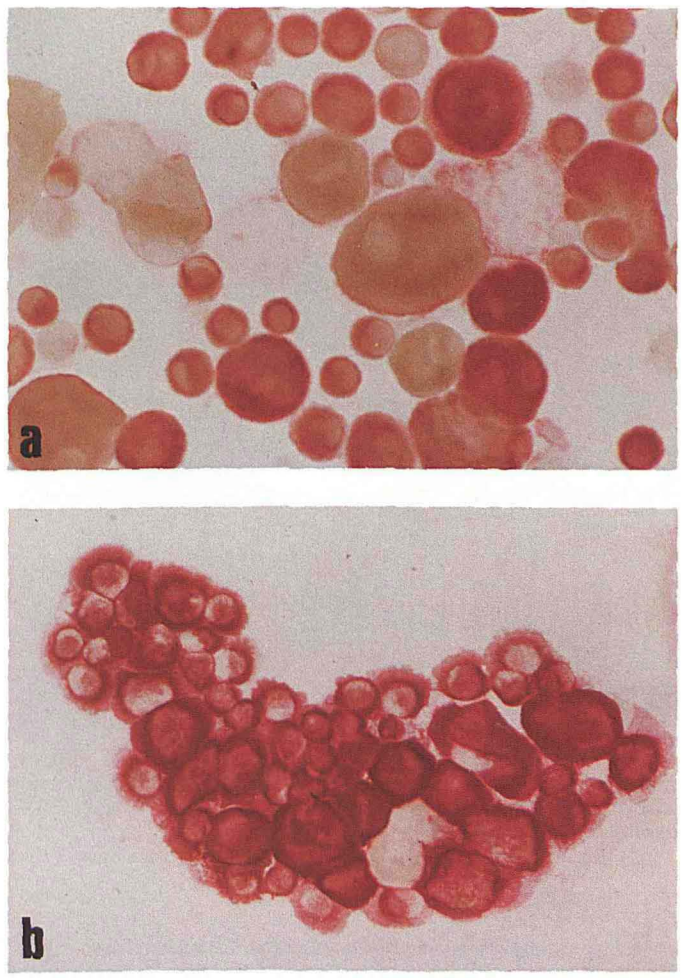

Figure 3. Cytocentrifuge preparation of cultured BCC cells and nKC stained with MoAb PKK2 (APAAP, 1:200). *a)* More than 76% of small BCC cells are stained, whereas only 51–75% of middle and 6–25% of large cells are stained (magnification ×360). *b)* nKC are homogeneously and strongly stained (magnification ×360).

immunoblot. K13, K15, and K19 were not detectable (Fig 8*a,c*). In particular, K5 and K6 of the neutral-to-basic subfamily and K14, K16, and K17 of the acidic subfamily were detected. In comparison, cultured human keratinocytes displayed keratin profiles (Fig 8*b,d*) showing the expression of K5, K6, K14, K16, and K17 but also K13, K15, and K19 [19,25,26].

Agar Suspension Culture The first cellular outgrowth in soft agar gels was observed in 27 of 30 BCC cell suspensions after 4–5 d. Whereas no outgrowth was observed from normal epidermal cells. BCC cells proliferated gradually and radiated into the gel in all directions (Fig 9).

DISCUSSION

Despite its high incidence, the growth characteristics of basal cell carcinoma in vivo are not fully clarified, and little is known about its behaviour in vitro. Söltz-Szöts [27] first succeeded in establishing explant cultures of BCC; however, the cells showed only a short-term survival. Kubilus et al [28] reported on the culture of nodular BCC in the presence of choleratoxin in the medium, although even then the success rate remained rather low. As a reason, they mentioned a lower plating efficiency of single cell suspensions of BCC cells compared to normal keratinocytes. Hernandez et al [9] established epithelial cell colonies from about 50% of cultured nodular BCC using tissue-culture plates coated with

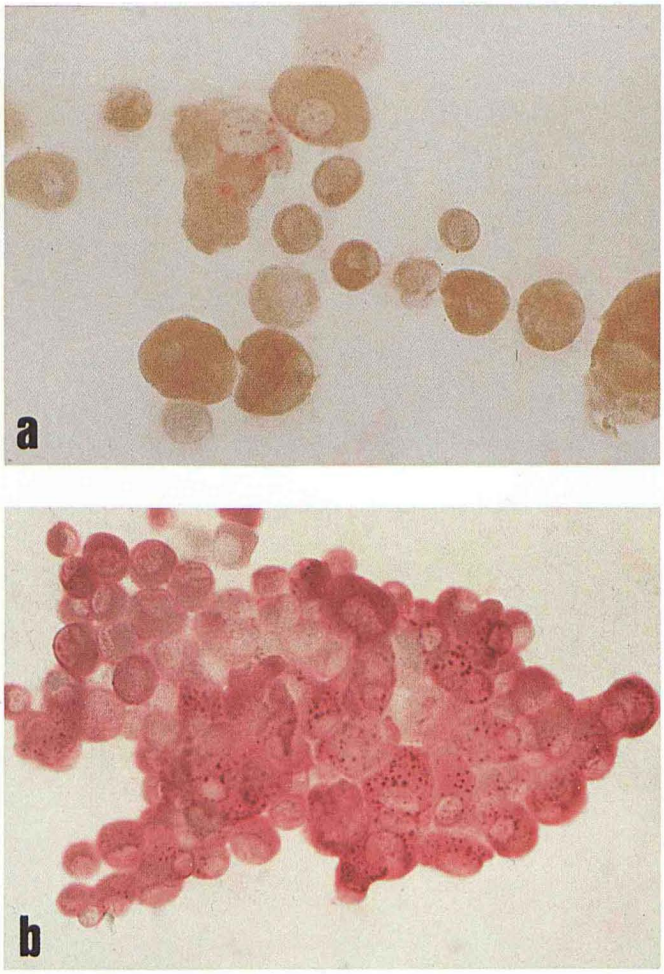

Figure 4. Cytocentrifuge preparation of cultured BCC cells and nKC stained with MoAb Filaggrin (APAAP, 1:100). *a)* BCC cells show no remarkable staining (magnification ×360). *b)* In nKC, positively stained keratohyaline granules are observed (magnification ×360).

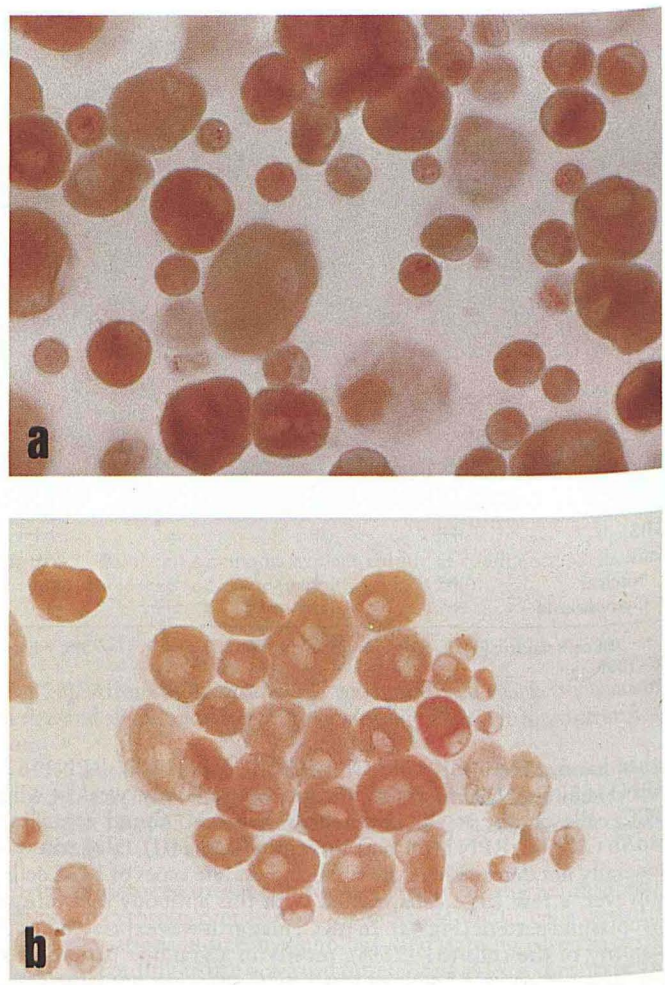

Figure 5. Cytocentrifuge preparation of cultured BCC cells and nKC stained with MoAb Ki67 (APAAP, 1:40). *a)* Dot-like nuclear staining is observed in 26–50% of small BCC cells, whereas cytoplasmic staining is observed in 51–75% of large cells (magnification ×360). *b)* Positive reactions of both nucleus and cytoplasm are remarkably less observed in nKC than in BCC (magnification ×360).

type IV collagen, which enhances the attachment of the cultured cells.

In our study, we obtained cell growth in 35 of 48 BCC (73%) by using explant cultures on 3T3 cell feeder layers. We could easily isolate the cells from "capsulated" solid BCC tumors macroscopically, and tumor nodules could be isolated under the stereomicroscope using fine needle dissection. For the enhancement of plating efficiency of BCC cells, we used feeder layers of mitomycin C–treated 3T3 cells. Additionally, the medium containing both human serum and cholera toxin may have enhanced the growth of BCC cells by elevating cyclic AMP [28]; human serum acts as a buffer and facilitates the attachment of the cultured cells by its numerous growth factors [29]. The fact that isolated cells from BCC grew in soft agar, although nKC did not, clearly reveals the neoplastic potential of BCC cells [30].

Based on the techniques used it seems likely that distinct differences occur between BCC cells and nKC in vitro regarding their morphology, growth rates, and expression of keratins and of several other antigens. Initially, outgrowing cells of BCC showed a spindle-shaped morphology and lacked the regular arrangement of nKC, which has also been observed by others [10]. At confluence, BCC cells in culture showed a large variation in size and shape, as compared to nKC.

The ultrastructural evaluation of cultured BCC cells after 16 d showed a stratified epithelium composed of five to six layers. However, in contrast to normal human keratinocytes cultured on 3T3 cell feeder layers [31], hemidesmosomes or regular desmosomes were only rarely detectable and cell-cell contacts were mainly established by tight junctions. Formation of a stratified in vitro epithelium by BCC cells without detection of hemidesmosomes has also been reported earlier [8,28]. The frequent observation of tight junctions, the strong accumulation of tonofilaments in the cytoplasm, and the lack of keratohyalin granules is in accordance with the ultrastructural features described in primary BCC explant cultures [8], whereas Kubilus et al [28] detected some keratohyalin granules in primary BCC suspension cultures. These differences are obviously due to their differing culture conditions, as we could not detect keratohyalin granules in primary nKC cultures.

The polymorphism of the cultured BCC cells, observed under the phase-contrast microscope, together with marked differences concerning the immunohistochemical staining pattern, led us to the discrimination of at least three cell types: a small, a middle sized, and a large type of BCC cells. These cell types reacted differently with MoAb, and the immunohistochemical results thus were regarded separately.

Because keratins are sensitive molecular markers for the different routes and stages of epithelial differentiation, the presence of the keratin pair K5/K14 in BCC cells manifests the origin of these cells as being derived from keratinocytes, the major cell type of stratified epithelia [32–34]. Accordingly, expression of K6, K16, and K17

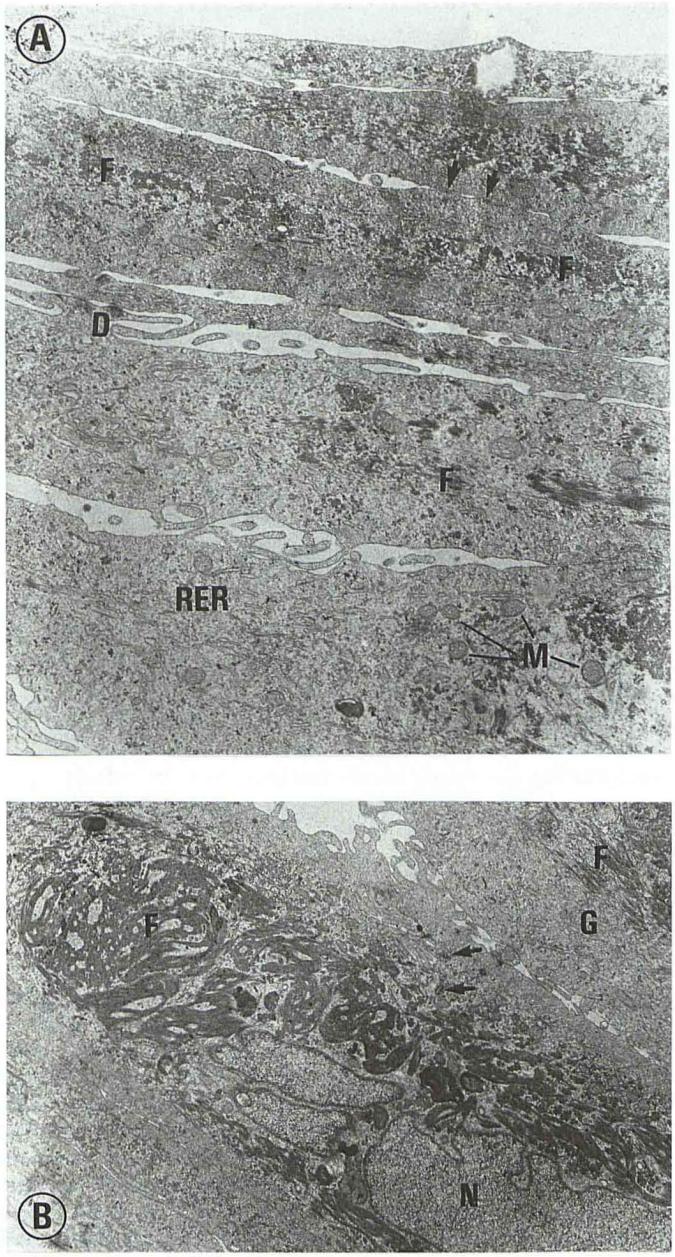

Figure 6. Electron micrographs of 16-day-old primary BCC cultures. *A.* Multilayered epithelium with regular cell organelles (RER, rough endoplasmic reticulum; M, mitochondria; F, tonofilaments). Sparse formation of incomplete desmosomes (D); intercellular contacts are mainly established by tight junctions *(arrows)*. Magnification ×14,065. *B.* BCC cells with irregularly arranged bundles of tonofilaments (F) were frequently observed in the intermediate layers, whereas desmosomes were only rarely detected *(arrows)*. N, nucleus; G, Goldi apparatus. Magnification ×15,646.

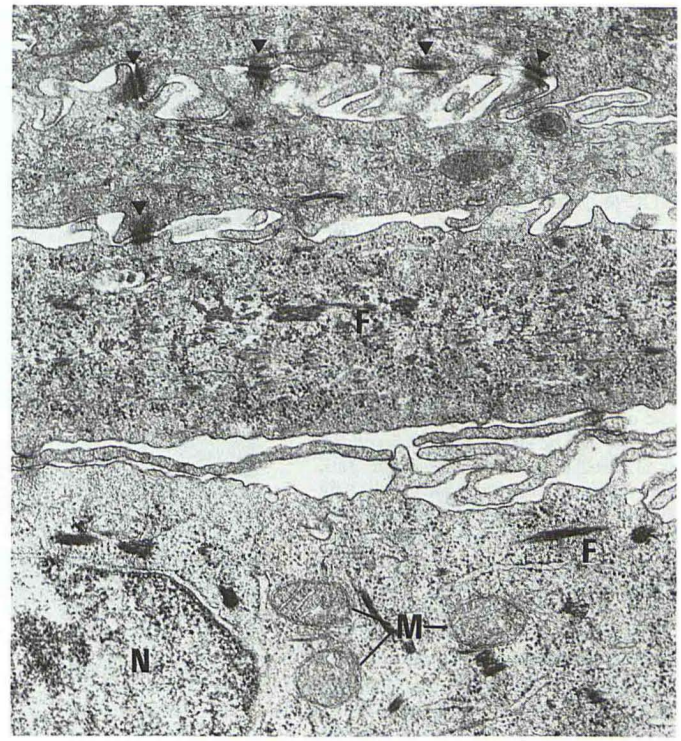

Figure 7. Ultrastructural aspect of the lower part of a 16-day-old primary nKC culture. Stratified epithelium with regular cell organelles including several intercellular desmosomes *(arrowheads)*. N, nucleus; M, mitochondria; F, tonofilaments (magnification ×28,896).

may reflect the degree of cell proliferation in these cells. Simple epithelial keratins such as K7, K8, and K18 were not detectable, a finding also supported by the immunohistochemical findings showing no reaction with MoAb RPN1160 (K18), RPN1162 (K7), and RPN1166 (K8). BCC cells in culture, however, lack the expression of keratins K13, K15, and K19. The presence of these keratins has been implicated to indicate an embryonic phenotype of keratinocyte differentiation [19]. Hernandez et al [9] reported in their study using one-dimensional gel electrophoresis that although nKC produced K1 when cultured with delipidized fetal calf serum, BCC cells failed to produce K1 even when cultured under the same conditions.

This may reflect the apparent plasticity of differentiation pathways in normal epidermal keratinocytes in culture. In contrast, cultured BCC cells appear to be more restricted in their pattern of keratin expression, possibly as a result of malignant transformation that is maintained in cell culture. The large cells showed an overall weaker reaction to MoAb than small and medium-sized cells, possibly due to a lower stage of maturation.

Staining with MoAb antifilaggrin was rarely and weakly detected only in large BCC cells, whereas a strong reaction with granular pattern was seen in nKC. This indicates mainly profilaggrin [35]. Because normal human keratinocytes display a more mature keratinization pattern under identical culture conditions [31], the behavior of BCC cells in vitro may reflect an intrinsic defect leading to incomplete keratinization similar to the in vivo situation [36]. This is also supported by the different keratin expression pattern of BCC cells found in vitro.

Laminin and GB3 are basement membrane proteins and known to play a role in epithelial adhesion to type IV collagen [37]. Nelson et al reported that nests of BCC in vivo are surrounded by laminin [38]. Our immunohistochemical results showed that 26–50% of small BCC cells synthesized laminin and GB3, whereas on middle or large BCC cells the reaction was weak or not detectable. In contrast, nKC showed strong reaction with these MoAb. The low plating efficiency of BCC cells [28] may be attributed to the low synthesis of these adhesion proteins.

Vimentin, which has been detected in large quantities in cultured nKC, could rarely be found in cultured BCC cells and, if so, only in the small cell type. Regarding the broad discussion about the significance of vimentin expression in cultured keratinocytes [39], we speculate that these findings reflect the slow growth and the altered differentiation of BCC cells in vitro, as compared to normal keratinocytes. Our immunohistochemical results may indicate that the small cell type represents a more basaloid BCC cell with stronger expression of cytokeratins and adhesion proteins, whereas the large type stands for a more immature, neoplastic cell, lacking completely

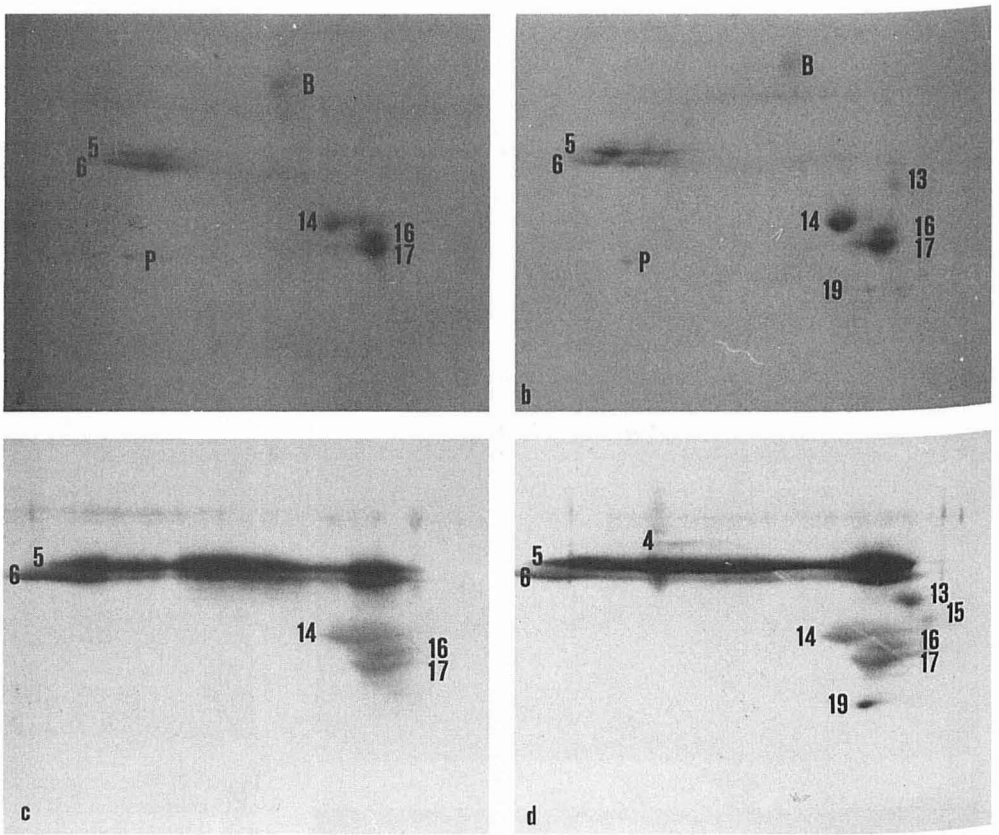

Figure 8. Keratin expression in cultured BCC and nKC cells at confluence of a primary culture. Two-dimensional separation by NEpHG/SDS-PAGE of BCC *(a,c)* and nKC *(b,c)*. Staining with Coomassie Blue *(a,b)* or labeling with anti-keratin antibodies on corresponding immunoblots *(c,d)*. The blots were sequentially incubated with the following antibodies: CK 4.62 detecting K19 only in nKC cells *(d)*, 10-2/2 detecting the type II keratins, and 8-2/4 detecting the type I keratins thereby establishing the complete keratin pattern as shown in *c* and *d*. The streaking of K5 and K6 is an artifact of these particular gels, the unlabeled spot migrating slightly slower than K13 corresponds to vimentin. Co-electrophoresed marker polypeptides are bovine serum albumin (B; apparent molecular weight 68 kD; pH 6.35) and yeast phosphoglycerol kinase (P; apparent molecular weight 43 kD; pH 7.4). Keratins are numbered according to the catalogue of human cytokeratins (Moll [32]); basic polypeptides are on the *left*.

the expression of laminin and GB3. The slight expression of filaggrin/profilaggrin only in the large BCC cell type does not contradict this view; however, cultures with separated clones of each cell type will provide further information.

Concerning the slow growth of BCC, Weinstein and Frost [40] studied BCC in vivo by autoradiographic techniques using tritiated thymidine and reported prolongation of the S-phase in BCC, as compared to normal epidermis. Also Heenen and Galand documented a prolonged S-phase in BCC using an in vitro model [41]. MoAb Ki67 detects a nuclear antigen that is present in the cell cycle of S, G_1, G_2, and M phases, but is absent in the G_0 phase [42]. In addition to the nuclear staining pattern, cytoplasmic staining has also been reported, possibly due to a cross-reaction with an unknown cytoplasmic epitope or to diffusion processes of stained particles into the cytoplasm [41]. With MoAb Ki67, a strong nuclear reaction was observed in small BCC cells, indicating a high cycling cell rate, whereas large BCC cells showed weak nuclear, but strong cytoplasmic, reactions. This observation is not yet fully understood, but could correlate to the peculiar growth pattern of BCC in vivo. The reduced growth rate and high cycling cell rate of BCC cells observed in our model may be explained by a prolongation of the S phase, suggesting that BCC cells in vitro retained some of their in vivo characteristics.

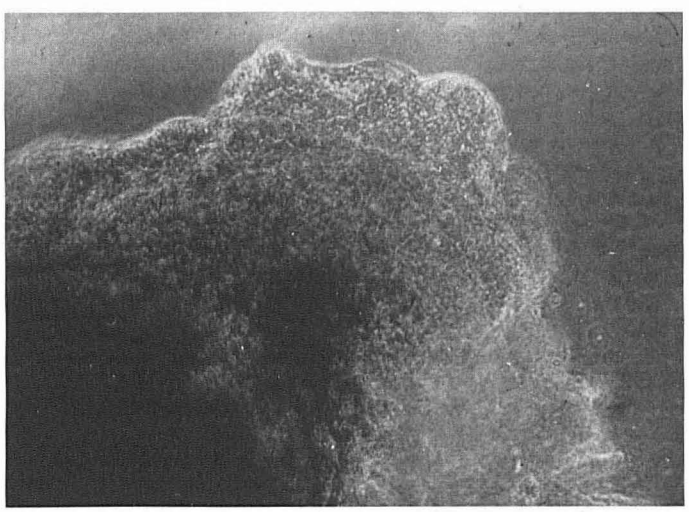

Figure 9. Isolated BCC nodules in soft agar culture after 10 d. Outgrowth of epithelial cells from fragmented BCC are observed (magnification ×100).

In conclusion, BCC cells have been successfully grown in vitro using a simple feeder-layer technique. Cultured BCC cells showed characteristic and reproducible patterns of growth, keratin expression, and ultrastructural differentiation that clearly distinguish them from epidermal basal cells. In culture, BCC cells displayed at least three cell types probably representing different stages of maturity. Additional experiments using this model and comparing cultured BCC cells with cells from the epidermal appendages (e.g., hair follicle) may enable us to gain further insights into the cellular origin of BCC.

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